BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 45, Issue of November 8, pp. 28271–28276, 1996
Printed in U.S.A.

Cloning of the Lipooligosaccharide α-2,3-Sialyltransferase from the Bacterial Pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae**

(Received for publication, August 6, 1996)

Michel Gilbert, David C. Watson, Anna-Maria Cunningham, Michael P. Jennings‡§, N. Martin Young, and Warren W. Wakarchuk¶

From the Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada and the ‡Molecular Infectious Diseases Group and Department of Paediatrics, Institute for Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, United Kingdom

The genes encoding the α-2,3-sialyltransferases involved in lipooligosaccharide biosynthesis from *Neisseria meningitidis* and *Neisseria gonorrhoeae* have been cloned and expressed in *Escherichia coli*. A high sensitivity enzyme assay using a synthetic fluorescent glycosyltransferase acceptor and capillary electrophoresis was used to screen a genomic library of *N. meningitidis* MC58 L3 in a "divide and conquer" strategy. The gene, denoted *lst*, was found on a 2.0-kilobase fragment of DNA, and its sequence was determined and then used to design probes to amplify and subsequently clone the corresponding *lst* genes from *N. meningitidis* 406Y L3, *N. meningitidis* M982B L7, and *N. gonorrhoeae* F62. Functional sialyltransferase was produced from the genes derived from both L3 *N. meningitidis* strains and the *N. gonorrhoeae* F62. However, the *N. meningitidis* M982B L7 gene contained a frameshift mutation that renders it inactive. The expression of the *lst* gene was easily detected using the enzyme assay, and the protein expression could be detected when an immunodetection tag was added to the COOH-terminal end of the protein. Using the synthetic acceptor *N*-acetyllactosamine-aminophenyl-(6-(5-(fluorescein-carboxamido)-hexanoic acid amide), the α-2,3 specificity of the enzyme was confirmed by NMR examination of the reaction product. The enzyme could also use synthetic acceptors with lactose or galactose as the saccharide portion. This study is the first example of the cloning, expression, and examination of α-2,3-sialyltransferase activity from a bacterial source.

Mammalian oligosaccharides containing terminal *N*-acetylneuraminic acid (Neu5Ac)[1] residues are recognized as biologically important carbohydrates for their function as receptors for lectins involved in cellular adhesion, as receptors for toxins, and for certain viruses (1). Some pathogenic bacteria have also been shown to carry sialylated oligosaccharides in their lipooligosaccharides (LOS), which are identical in structure to those found in mammalian glycolipids. *Neisseria gonorrhoeae* and *Neisseria meningitidis* LOS contain α-2,3-monosialylated lacto-*N*-neotetraose (2, 3), and in *Camplyobacter jejuni* the structures are variants of a mono-, di-, or tri-sialylated ganglioside (4). The role of these sialylated oligosaccharides in the pathogenesis of *N. gonorrhoeae* has been clearly demonstrated (2), and although the precise role of similar sialylated LOS in the pathogenesis of *N. meningitidis* or *C. jejuni* is not known, it is presumed to be a form of molecular mimicry that aids in the evasion of the host immune response.

There have been extensive studies of the sialyltransferases involved in mammalian glycoconjugate synthesis, where at least eight different enzymes with an α-2,3-sialyltransferase activity have been examined either through protein purification or the cloning of the genes (1). In contrast, the bacterial sialyltransferases involved in the synthesis of α-2,3-sialylated lacto-*N*-neotetraose from *N. gonorrhoeae* or *N. meningitidis* and the enzyme(s) involved in the sialylation of lipopolysaccharide from *C. jejuni* have not been purified nor their genes cloned to date. There have been several reports of measurement of the sialyltransferase activity in *Neisseria* species (3, 5), and the enzyme uses the same sugar nucleotide donor, CMP-Neu5Ac, as all the mammalian enzymes. They also appear to be solubilized by Triton X-100, which suggests a membrane association (5, 6), and to be low abundance proteins that so far have eluded purification to homogeneity. Attempts have been made to clone these prokaryotic sialyltransferases using nucleic acid probes based on the mammalian "sialyl motif" or using complete mammalian genes (Ref. 3 and references within), but none of these attempts has revealed a sialyltransferase gene. Recently *N. gonorrhoeae* mutants defective in LOS sialylation have been described (7), but these have not so far enabled the structural gene for the LOS sialyltransferase to be identified.

* Part of this work was performed in the laboratory of Dr. E. R. Moxon, which was in part supported by programme grants from the Medical Research Council (UK) and the Wellcome Trust and Action Research. This is National Research Council of Canada Publication Number 39527. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U60660, U60661, U60663, and U60664.

§ Recipient of a Beit Memorial Research fellowship. Present address: School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Kessels Rd., Nathan 4111, Queensland, Australia

¶ To whom correspondence should be addressed: Inst. for Biological Sciences, National Research Council of Canada, 100 Sussex Dr., Ottawa, ON K1A 0R6, Canada. Tel.: 613-990-0817; Fax: 613-941-1327; E-mail: wakarchu@biologysx.lan.nrc.ca.

[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; LOS, lipooligosaccharide; CE, capillary electrophoresis; FCHASE, 6-(5-fluorescein-carboxamido)-hexanoic acid succimidyl ester; FCHASE-LacNAc, aminophenyl-*N*-acetyllactosamine-6-(5-(fluorescein-carboxamido)-hexanoic acid amide); FCHASE-Lac, aminophenyl-lactose-6-(5-fluorescein-carboxamido)-hexanoic acid amide; PCR, polymerase chain reaction; kb, kilobase; MES, 2-(*N*-morpholino)ethanesulfonic acid pfu, plaque-forming unit; PAGE, polyacrylamide gel electrophoresis; NOE, nuclear Overhauser effect.

EXHIBIT G

Here we describe the first cloning and characterization of a CMP-Neu5Ac:β-galactoside α-2,3-sialyltransferase from the pathogens *N. meningitidis* and *N. gonorrhoeae* achieved by the use of a highly sensitive screening procedure based on the expression of enzyme activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The following *N. meningitidis* strains were used in this study: immunotype L3 MC58 (NRCC 4728); immunotype L3 406Y (NRCC 4030); and immunotype L7 M982B (NRCC 4725). DNA from *N. gonorrhoeae* F62 (ATCC 33084) was a kind gift from Dr. Wendy Johnson (Health Canada, Ottawa, ON, Canada).

Basic Recombinant DNA Methods—Plasmid DNA isolation, restriction enzyme digestions, purification of DNA fragments for cloning, ligations, and transformations, and DNA sequencing were performed as recommended by the enzyme supplier or the manufacturer of the kit used for the particular procedure. PCR was performed with *Pwo* polymerase as described by the manufacturer (Boehringer Mannheim). Restriction and DNA modification enzymes were purchased from New England Biolabs Ltd. (Mississauga ON, Canada). Qiaprep columns were from Qiagen Inc. (Chatsworth, CA). DNA sequencing was performed with an Applied Biosystems (Montreal, Canada) model 370A automated DNA sequencer using the manufacturer's cycle sequencing kit. DNA sequence analysis and protein alignments were performed with the Genetics Computer Group suite of programs (Madison, WI).

Cloning and Sequencing of the Sialyltransferase from N. meningitidis—The genomic library was prepared using 3–5-kb fragments from a *Hae*III partial digest of the chromosomal DNA of *N. meningitidis* MC58 into λZAPII (Stratagene, La Jolla, CA) as the vector (8). The λZAPII library was plated at low density, and 3600-well isolated plaques were picked in pools of 100. Phage suspensions were made as described previously (9) and used to infect 1.5-ml cultures of *E. coli* XL1-Blue (in LB medium with 0.2% maltose, 10 mM $MgSO_4$, and 2 mM isopropyl-1-thio-β-D-galactopyranoside), which were grown for 4.5 h. Toluene was added to 1%, and the cells were then assayed for sialyltransferase activity as described below. The positive pools were plated, and then plaques were picked in pools of five and analyzed again for activity. Positive pools of five were then used to isolate individual clones expressing sialyltransferase activity. Phagemids carrying the sialyltransferase gene were excised from the positive λZAPII clones using the ExAssist helper phage and the SOLR *E. coli* strain as described by the supplier Stratagene. The DNA sequence for the 2.0-kb insert of this first plasmid (pNST-01) was determined, and PCR primers based on this sequence were used to amplify the genes from DNA prepared from *N. meningitidis* 406Y, M982B, and *N. gonorrhoeae* F62. The primer sequences were 5′ primer SIALM-5F, (nucleotides 540–569 in pNST-01 insert sequence, *Nde*I site shown in bold italics) 43-mer: 5′-CTTAGGAGGT-***CATATG***TTCAATTTGTCGGAATGGAGTTTTAGG-3′, and 3′ primer SIALM-16R, (nucleotides 1688–1658 of pNST-01 insert sequence, *Sal*I site shown in bold italics) 42-mer: 5′-CCTAG***GTCGAC***TCATTAA TTTTTATCGTCAAATGTCAAAATC-3′.

Detection of the Sialyltransferase by Western Blotting—The gene product was detected in *E. coli* by first constructing a plasmid consisting of the *lst* open reading frame from pNST-01 and the peptide tag for immunodetection with anti-*c-myc* antibody as described previously (10). This construct was made using the following primers for PCR amplification, 5′ end primer was the standard M13 "reverse" primer, and 3′ end primer SIALM-18R (*Sal*I site in italics, and the c-*myc* tag in bold): 5′-CCTAG***GTCGAC***TCATTAGTTCAGGTCTTCTTCGCTGATCAG-TTTTTGTTCATTTTTATCGTCAAATGTCAAAATCGGG-3′ 78-mer. The PCR product was cloned in the vector pT7–7 (11), and protein expression was then induced with isopropyl-1-thio-β-D-galactopyranoside. Western blotting was performed as described previously (10).

Measurement of Sialyltransferase Activity—The sialyltransferase activity from *N. meningitidis* MC58 L3, 406Y L3, and M982B L7 and *E. coli* carrying pNST plasmids was measured in toluene-treated cells or cell-free extracts prepared as described previously (12). The sialyltransferase acceptors were derived from aminophenylglycosides reacted with 6(5-fluorescein-carboxamido)-hexanoic acid succimidyl ester (FCHASE) and were prepared as described previously (12). Reactions for the enzyme were performed at 37 °C in 20 μl of 50 mM MES buffer, pH 6.0, 10 mM $MnCl_2$, with 0.2 or 1.0 mM labeled acceptor, 0.2 mM CMP-Neu5Ac donor, and various amounts of enzyme, from either crude bacterial extracts or extracts of recombinant *E. coli* with the cloned gene. The recombinant enzymes were assayed for 10–120 min, whereas extracts from *N. meningitidis* were incubated 1–15 h. The reactions were terminated by diluting the reaction 1:100 with 10 mM NaOH. These samples were then diluted appropriately in water prior to analysis by capillary electrophoresis.

Capillary electrophoresis (CE) was performed with a Beckman (Fullerton, CA) P/ACE 5510 equipped with a 3 mW Argon-ion laser-induced fluorescence detector (λ excitation = 488 nm; λ emission = 520 nm). The capillary was bare silica 75 μm × 47 cm, with the detector at 40 cm. The capillary was conditioned before each run by washing with 0.2 M NaOH for 2 min, water for 2 min, and 25 mM sodium tetraborate, pH 9.4, for 2 min. Samples were introduced by pressure injection for 2–5 s, and the separation was performed at 15 kV, 75 μA. Peak integration was performed with the Beckman System Gold (version 8.1) software.

For rapid detection of enzyme activity, samples from the transferase reaction mixtures were examined by thin layer chromatography on silica-60 TLC plates (Merck). A spot of 0.5–1.0 μl from the reaction was air dried, and the plate was developed with ethyl acetate/methanol/water/acetic acid (7:2:1:0.1). After drying, the acceptor and product spots could be seen by illumination of the plate with a 365 nm UV lamp. The product R_f under these conditions was 0.05.

Preparative Sialyltransferase Reactions—Preparative enzyme reactions were performed as coupled enzyme reactions with the cloned *N. meningitidis* CMP-Neu5Ac synthetase.[2] The reactions contained 25 mM HEPES, pH 7.5, 0.2 mM dithiothreitol, and 10 mM $MgCl_2$, 400 milliunits/ml of CMP-Neu5Ac synthetase, 300 milliunits/ml inorganic pyrophosphatase (Sigma), 1.5 mM CTP, 1.5 mM Neu5Ac, and 50 milliunits of sialyltransferase (based on FCHASE-aminophenyl-LacNAc as the acceptor). The acceptor, FCHASE-aminophenyl-Lac or FCHASE-aminophenyl-LacNAc, was dried down in the tube under vacuum, and the reagents were then added to the tube; the concentration of FCHASE-aminophenylglycoside in these reactions was 1 mM. These reactions were performed at 30 °C for 3–5 h. After the reaction, the FCHASE-aminophenylglycoside was bound to a Sep-Pak C18 reverse phase cartridge (Waters, Milford, MA), desalted by washing with water, and then eluted in 50% acetonitrile.

Determination of the Linkage Specificity of the Sialyltransferase—The product from a preparative sialyltransferase reaction was examined by NMR. Samples for NMR were prepared by the TLC method and were then freeze dried from D_2O three times prior to collection of the spectra. Prior to lyophilization and exchange with D_2O, the pH of the sample was adjusted to 7. NMR data collection was performed with a Bruker AMX 600 spectrometer. Spectra were recorded at 300 K in 5-mm tubes at a concentration of 0.5 mg of FCHASE-aminophenylglycoside in 0.6 ml of D_2O. Chemical shifts in ppm are relative to the methyl resonance of acetone set at 2.225 ppm for ^1H and 31.07 ppm for ^{13}C. All NMR experiments and spectral analysis were performed as described previously (13).

RESULTS

Detection and Characterization of α-2,3-Sialyltransferase Activity from N. meningitidis—The initial part of this work was performed with the *N. meningitidis* strain 406Y L3, which possesses an LOS identical to that of strain MC58 but has a different capsular type. Both of these strains elaborate the L3 immunotype LOS, which consists of a lacto-*N*-neotetraose branch with an α-2,3-sialic acid on the terminal galactose residue (13). Both of these strains produced easily detectable levels of α-2,3-sialyltransferase when using as little as a single colony (10^7 cells) with the CE-based assay. Crude extract from *N. meningitidis* 406Y L3 was used to prepare material for determination of the linkage of the sialoside being synthesized and the enzyme was verified by NMR of its product to be a β-galactoside α-2,3-sialyltransferase. By NMR, the complete ^1H assignment of the compounds was performed. It was found that the ^1H chemical shifts (Table I) were similar to those of reported structures containing α-NeuAc-(2-3)-Gal (13). Also an NOE across the glycosidic bond $H3_{ax}$-sialic acid to H3-Gal confirmed that the β-galactoside α-NeuAc-(2-3)-Gal linkage was present in the product (Fig. 1).

Variation of the reaction conditions showed that the enzyme had a pH optimum of 6.0 and that the activity was stimulated 2-fold by the addition of either 10 mM $MgCl_2$ or 3-fold by 10 mM $MnCl_2$. However, there are no stringent metal requirements

[2] M. Gilbert and W. Wakarchuk, unpublished data.

TABLE I
NMR chemical shifts for α-D-Neu5Ac-(2–3)-β-D-Galp-(1–4)-β-D-GlcpNAc-aminophenyl-(6–5-(fluorescein-carboxamido)-hexanoic acid amide)

^1H at 600 MHz, sample in D_2O, pH 7, 300 K, chemical shifts in ppm are relative to the methyl resonance of acetone (2.225 ppm for ^1H and 31.07 for ^{13}C). ^1H chemical shifts obtained from two-dimensional data (+/−0.08 ppm). ^{13}C chemical shift obtained from HMQC spectrum (+/−0.8 ppm). The CH_2 groups of hexanoic acid amide ($NHCO-(CH_2)_5-NH$) have ^1H chemical shifts at 3.49, 1.73, 1.49, 1.77, and 2.43 ppm and respective ^{13}C signals at 40.6, 28.8, 26.0, 25.7, and 37.1 ppm. The aminophenyl HC = CH ^1H signals are at 6.93 and 7.26 ppm with their respective ^{13}C signals at 118.3 and 125.3 ppm. The three ^1H AMX spin systems for the fluorescein carboxamido group with J_{AM} = 8–9 Hz and J_{MX} = 2 Hz are at (7.13, 6.65, and 6.72), (7.11, 6.67, and 6.72) and (7.26, 7.81, and 8.17) ppm. Their respective ^{13}C signals are at (132.2, 124.1, and 104.5), (132.7, 124.1, and 104.5) and (125.3, 129.0, and 128.0) ppm.

Sugar	Position	H	C
GlcNAc	1	4.87	100.2
	2	3.89	55.6
	3	3.71	73.0
	4	3.71	79.3
	5	3.49	75.6
	6	3.88	60.7
	6′	3.71	
	NAc	1.94	22.8
Gal	1	4.52	103.4
	2	3.59	70.2
	3	4.12	76.5
	4	3.97	68.3
	5	3.73	76.1
	6	3.77	61.9
	6′	3.73	
Neu5Ac	3_{ax}	1.82	40.6
	3_{eq}	2.77	
	4	3.69	69.4
	5	3.85	52.4
	6	3.64	73.5
	7	3.56	69.0
	8	3.9	72.6
	9	3.88	63.5
	9′	3.71	
	NAc	2.03	22.8

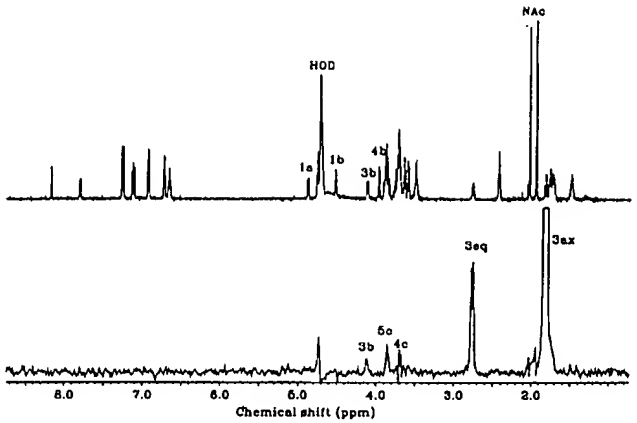

FIG. 1. **^1H NMR spectrum of sialylated FCHASE-aminophenyl-*N*-acetyllactosamine.** *1a* refers to H1 of GlcNAc; *1b*, *3b*, and *4b* denote the H1, H3, and H4 resonances of Gal; and *3eq*, *3ax*, *4c*, and *5c* represent Neu5Ac signals. The NOE spectrum (*bottom panel*) taken from the two-dimensional NOE spectrum shows the NOE from $H3_{ax}$ of sialic acid to H3 of Gal due to the presence of the Neu5Ac(2–3)Gal linkage in the final product.

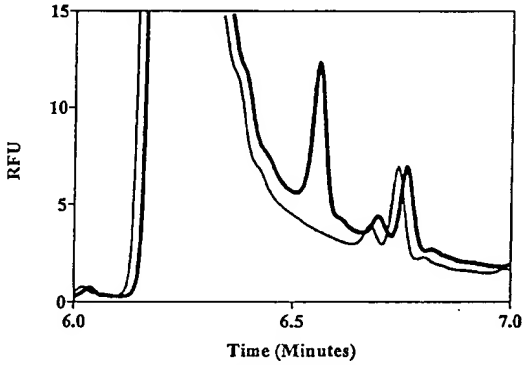

FIG. 2. **Detection of activity of the recombinant *N. meningitidis* α-2,3-sialyltransferase.** Two electropherograms are superimposed here to illustrate the level of sialyltransferase activity found in a 1.5-ml *E. coli* culture infected with 1000 pfu from the genomic DNA bank of *N. meningitidis* MC58 in λZAPII. The *thin line* is from a run where the reaction contained no CMP-Neu5Ac donor, and the *thick line* is from a run containing the CMP-Neu5Ac donor. The peak at 6.6 min was shown to comigrate with FCHASE-α-2,3-sialyl-*N*-acetyllactosamine. The detector response is expressed in relative fluorescence units (*RFU*).

because it was active in the presence of 5 mM EDTA. These same conditions were also optimal for the enzyme from crude extracts of MC58, 406Y, and for the recombinant enzymes from MC58. The natural enzyme was mostly associated with the cell membrane fraction (86% in the cell membrane pellet after centrifugation at 100,000 × *g*). However, no detergent was required for activity, and in fact many common detergents tested inhibited the enzyme, with the exception of Triton X-100 up to 0.2%. Using this method no activity could be detected in M982B L7 cells.

Cloning and Sequencing of the Sialyltransferase Gene from N. meningitidis MC58—Using the CE laser-induced fluorescence assay, we observed sialyltransferase activity one time out of five when we infected a 1.5-ml isopropyl-1-thio-β-D-galactopyranoside-induced *E. coli* XL1-Blue culture with 1000 pfu from the *N. meningitidis* MC58 genomic library in λZAPII (Fig. 2). Formation of the product peak in the electropherogram required the addition of CMP-Neu5Ac, and it migrated the same as the sialidase-sensitive product peak formed by the natural enzyme. The peak in the CE electropherogram corresponds to 20 attomoles (2×10^{-17} mol) of product. Single clones expressing the sialyltransferase were obtained by a "divide and conquer" strategy sequentially screening pools of 100 pfu from the λZAPII library of MC58, pools of 5 pfu derived from the first positive pool, and finally individual plaques plated at low density. The initial screening yielded two positive pools of 100 pfu out of 36. From one of these pools we screened 60 pools of 5 pfu and obtained three positive pools. From the positive pools of 5 pfu we obtained many individual positive clones and the pBluescript SK^- phagemids excised from them were found to carry a 2.0-kb insert.

The 2.0-kb insert was sequenced on both strands (GenBank® accession number U60660) and a BLASTX search was performed in GenBank® in order to identify any homology with previously sequenced genes. This analysis revealed two partial open reading frames (nucleotides 1–140 and 1853–2039), located at the opposite ends of the 2.0-kb insert, that were clearly homologous with various bacterial isocitrate dehydrogenases (60–85% identity) and various bacterial cytochrome *c′* proteins (43–63% identity), respectively. A third open reading frame (nucleotides 573-1685) was designated *lst* (lipooligosaccharide sialyltransferase) and revealed significant homology to a *Haemophilus influenzae* gene designated *lsg-ORF2* (GenBank® accession number M94855). Pair-wise alignment between the translation products of *lst* and *lsg-ORF2* indicated that their amino acid sequences share 29.3% identity and 56.3% similarity (Fig. 3).

The *lst* gene has two potential start codons, and the second of these is more likely to be used because the amino acid sequence immediately following this start codon appears to be a non-

```
lst      MGLKKACLTVLCLIVFCFGIFYTFDRVNQGERNAVSLLKEKLFNEEGEP   49
lst      VNLIFCYTILQMKVAERIMAQHPGERFYVVLMSENRNEKYDYYFNQIKDK   99
lsg02    MNLILCCTPLQVLIARKIIELHPNEQFFGVMFGGVWDKKRTLYASKLAEV   50
lst      AERAYFFHLPYGLNKSFNFIPTMAELKVKSMLLPKVKRIYLASLEKVSIA  149
lsg02    CSDSMNIDTGKDL.KGFDSLKLMRQLKNK.ITHKGFDKVFLANLNSLWLQ   98
lst      AFLSTYPDAEIKTFDDGTGNLIQSSSYLGDEFSVNGTIKRNFARMMIGD.  198
lsg02    TYLSHVSFKELYTFDDGSDNIFPHPNLLREP....DTFKYKLIKAFIGDK  144
lst      WSIAKTRNASDEHYTIFKGLKNIMDDGRRKMTYLPLFDASELKTGDETGG  248
lsg02    YSVNKLFKKIKKHYTVYPNYKNIVSN....IEPISLWDN...QIDCEIDG  187
lst      TVRILLGSPDKEMKEISEKAAKNFK....IQYVAPHPRQTYGLSGVTTLN  294
lsg02    EVSFFIGQPLLNTKEENISLIKKLKDQIPFDYCFPHPAEDYRVDGVNYVE  237
lst      SPYVIEDYILREIKKNPHTRYEIYTFFSGAALTMKDFPNVHVYALKPASL  344
lsg02    SELIFEDYVFKHL...SNKKIIIYTFFSSVAFNLLSHPNVEI.RFIRTSI  283
lst      PEDYWLKPVYALFTQSGIPILTFDDKN     371
lsg02    P...RWQFCYDSFPDLGLTIYKEI        304
```

FIG. 3. **Protein alignment of the *N. meningitidis* MC58 *lst* and *H. influenzae lsg-02* translation products.** An alignment of the sequences using the GCG BESTFIT program is shown. The *solid lines* between the sequences show identical residues, and the *dotted lines* show similar residues.

```
F62      .....V.... .......... .......... .........D .......K.. .......... 60
L7       .S........ .......... .........H. .........D .......... .......... 60
406Y     .......... .......... .........H. .........D .......... .......... 60
MC58     MGLKKACLTV LCLIVFCFGI FYTFDRVNQG ERNAVSLLKE KLFNEEGEPV NLIFCYTILQ 60

F62      .......... .......... .......... .......... ......Y... .......... 120
L7       .......... .......... .......... ...K...... .......... .......... 120
406Y     .......... .......... .......... ...K...... .......... .......... 120
MC58     MKVAERIMAQ HPGERFYVVL MSENRNEKYD YYFNQIKDKA ERAYFFHLPY GLNKSFNFIP 120

F62      .......... .......... .......... .......... .......N.. .RE.....G. 180
L7       .......... .......... .......... .*                              151
406Y     .......... .......... .......... .......... .......... .......... 180
MC58     TMAELKVKSM LLPKVKRIYL ASLEKVSIAA FLSTYPDAEI KTFDDGTGNL IQSSSYLGDE 180

F62      .A...A.... .....V.... .......... .......... .......... .......... 240
406Y     .......... .......... .......... .......... .......... .......... 240
MC58     FSVNGTIKRN FARMMIGDMS IAETRMASDE HYTIFKGLKN IMDDGRRKMT YLPLFDASEL 240

F62      .A........ .......... .......... ..N....... .......... .A........ 300
406Y     .A........ .......... .......... ..N....... .......... .......... 300
MC58     KTGDETGGTV RILLGSPDKE MKEISEKAAK NFKIQYVAPH PRQTYGLSGV TTLNSPYVIE 300

F62      .......... .......... .......... .......... .......... .......R.A 360
406Y     .......... .......... .......... .......... .......... .......... 360
MC58     DYILREIKKN PHTRYEIYTF FSGAALTMKD FPNVHVYALK PASLPEDYWL KPVYALFTQS 360

F62      D......... .     371
406Y     .......... .     371
MC58     GIPILTFDDK N     371
```

FIG. 4. **Protein sequence alignment of the *lst* proteins from *N. meningitidis* and *N. gonorrhoeae*.** Comparison of the derived protein sequences of the *lst* genes from *N. meningitidis* MC58 L3, 406Y L3, and M982B L7 as well as from *N. gonorrhoeae* F62. The complete sequence of the MC58 L3 protein is presented, whereas only divergent residues are presented for the other sequences. The *lst* gene from M982B L7 encodes a truncated product of only 151 residues.

cleavable leader sequence (14), and a potentially very good ribosome binding site (AGGGA) occurs just upstream.

Comparison of Sialyltransferase Genes from Different N. meningitidis Strains and N. gonorrhoeae—Isolation of the genes from *N. meningitidis* 406Y L3 (GenBank® accession number U60661), M982B L7 (GenBank® accession number U60663), and *N. gonorrhoeae* F62 (GenBank® accession number U60664) was accomplished with PCR primers based on the gene from MC58 L3 (GenBank® accession U60660). We found 12 base differences, which results in five amino acid differences between the two genes from the L3 immunotype strains (Fig. 4), 19 differences in the gene from M982B L7 compared with MC58, and 12 differences in the M982B L7 sequence compared with that of 406Y L3. The gene from M982B L7 contains a frameshift mutation at nucleotide 454 and consequently would encode a truncated protein of only 151 amino acids (Fig. 4).

The gene from *N. gonorrhoeae* F62 shows 63 nucleotide differences compared with the *N. meningitidis* MC58, 62 nucleotide differences compared with the 406Y L3 gene, and 66 nucleotide differences compared with the M982B L7 gene. These differences in the DNA sequence of the *N. gonorrhoeae* F62 gene result in 16 and 17 amino acid differences in the protein when compared with the MC58 L3 and 406Y L3, respectively (Fig. 4).

Expression of the Sialyltransferase Gene—We could easily detect enzyme activity in *E. coli* carrying pNST plasmids, and this expression of the *lst* gene depended on the vector derived *lac* promoter because there was no detectable enzyme activity when the gene's orientation was inverted. There was at least 30-fold more enzyme activity from the pNST-01 containing clones compared with *N. meningitidis* L3 strains. However, the expression of the *lst* gene was not high enough to permit simple detection of an overexpressed protein by SDS-PAGE analysis. A plasmid was therefore constructed to introduce a c-*myc* immunodetection peptide tag at the COOH-terminal end of the protein. When this plasmid was used to express the *lst* gene, we could detect an immunoreactive protein with an M_r of 41,000 (Fig. 5), which is slightly shorter than the predicted size of the *lst* gene product including the c-*myc* peptide tag (expected M_r 44,000).

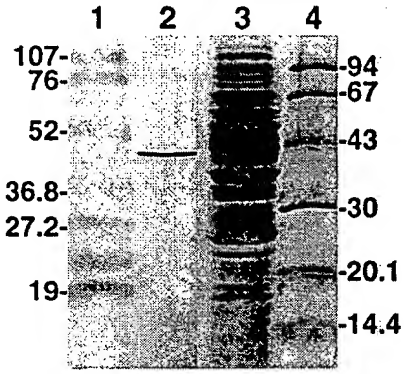

FIG. 5. **Western blot analysis of the *lst* gene product.** The *lst* gene with a c-*myc* immunodetection tag added at its COOH terminus was expressed in the plasmid pT7-7, and then a sample of the supernatant from freeze-thawed cells was subjected to SDS-PAGE followed by Western blotting onto polyvinylidene difluoride membrane and subsequent detection with anti-c-*myc* antibody. *Lane 1*, prestained M_r markers, with the $M_r \times 10^{-3}$ shown; *lane 2*, *E. coli* pNST-09 extract probed with the anti-c-*myc* antibody; *lane 3*, identical sample as in *lane 2*, but stained with Coomassie Blue; *lane 4*, M_r markers stained on the blot at the same time as *lane 3*.

Acceptor Specificity of the lst Sialyltransferase—The natural acceptor for this enzyme is a terminal *N*-acetyllactosamine sequence on the lacto-*N*-neotetraose branch of the LOS from L3 immunotypes. We found that the enzyme would use as acceptors the synthetic saccharides: FCHASE-aminophenyl-LacNAc, FCHASE-aminophenyl-Lac, and FCHASE-aminophenyl-Gal. To assess the relative acceptor specificities we assayed for activity using two different substrate concentrations and compared the specific activites of both the native MC58 sialyltransferase and the recombinant form (pNST-01). Comparison of the specific activity reveals a strong preference for the LacNAc containing acceptor (Table II).

DISCUSSION

The availability of a highly sensitive enzyme assay was instrumental in successful screening of clones expressing the *N.*

TABLE II

Comparison of acceptor specificities of N. meningitidis α-2,3-sialyltransferase

Two substrate concentrations were used to assay enzyme activity from both sources. The relative specific activities were calculated from the percent conversion of the substrate to product using the CE assay. The assays were performed so that similar percent conversions are compared for both sources of enzyme.

Enzyme source	*N*-acetyllactosamine[a]		Lactose		Galactose	
	0.2 mM	1.0 mM	0.2 mM	1.0 mM	0.2 mM	1.0 mM
MC58 L3	0.70[b]	1.45	0.11	0.45	0.006	0.033
pNST-01	23.9	46.8	4.2	15.4	0.17	0.84

[a] The acceptors used in this experiment were all FCHASE-aminophenyl-glycosides.
[b] The activity values are in milliunits/mg of protein.

meningitidis α-2,3-sialyltransferase. The assay uses a glycosyltransferase acceptor, which is easy to synthesize, and does not require specially constructed CE equipment as has been previously described for the ultrasensitive detection of glycosyltransferase reaction products (15). The acceptors used in this study were made from widely available glycosides, and fluorophores and the CE equipment used was commercially available. We were able to reliably detect attomole (10^{-18} mol) quantities of reaction products, which was more than adequate for screening for α-2,3-sialyltransferase expression.

The *lst* gene from MC58 L3 occurs between two genes unrelated to LOS synthesis, isocitrate dehydrogenase and cytochrome *c'*, and is not part of a LOS synthesis operon unlike other *N. meningitidis* LOS glycosyltransferases (8). This is similar to the situation with the *E. coli* and *N. meningitidis* α-2,8-polysialyltransferase involved in capsule biosynthesis, although these genes are adjacent to the CMP-Neu5Ac synthetase (16). It is interesting to speculate that the *lst* gene is found on its own as the result of a transposition event, although we have no evidence for insertion elements or transposon-like sequences flanking the gene. Sequence analysis and data base comparisons showed this gene to be distinct from both the mammalian α-2,3-sialyltransferase family, the bacterial α-2,8-sialyltransferase family, and the bacterial 3-deoxy-α-D-*manno*-octulosonic acid transferases, which transfer a related sugar also from a CMP donor. The *lst* gene product was, however, shown to be similar to the *lsg-ORF2* gene product from *H. influenzae*. Although *lsg-ORF2* has been demonstrated to be involved in LOS biosynthesis, it may not encode an α-2,3-sialyltransferase because it was reported to be involved in the expression of a Gal-GlcNAc LOS epitope (17). For the cloning of the *N. gonorrhoeae* gene, the F62 strain was used because it has been studied in relation to the role LOS sialylation plays in pathogenesis and because many LOS glycosyltransferases have been shown to be common to both species (8). An examination of the gene derived from *N. gonorrhoeae* F62 shows only a small number of differences, which is similar to other LOS biosynthesis gene comparisons from *N. meningitidis* and *N. gonorrhoeae* (8).

We have observed that the activity from *N. meningitidis* extracts is associated with the membrane fraction. The protein encoded by the *lst* gene appears to have an uncleavable signal peptide, and computer aided prediction programs suggest that the sialyltransferase is an integral inner membrane protein (14). However, the original papers describing the sialyltransferase activity from both *N. meningitidis* and *N. gonorrhoeae* suggest that the sialyltransferase would be an outer membrane protein on the basis that the enzyme activity is extracted from whole cells by Triton X-100 (5, 6). We have not yet fully purified the recombinant product, but the predicted size of the immunotagged protein is slighty larger than that we observed by SDS-PAGE. The difference between the observed and the expected M_r is less than 7% and is within the accuracy of SDS-PAGE. However, the possibility exists that the recombinant protein is truncated at the amino-terminal end, which would result in the loss of the predicted noncleavable leader sequence. Experiments are in progress to determine if the *lst* protein is expressed intact in *E. coli*.

That this gene functions in the sialylation of LOS is inferred from an examination of *N. meningitidis* M982B L7, which appears to be a natural sialyltransferase mutant. The sialyltransferase gene derived from this L7 strain contains a frameshift mutation at nucleotide 454 that renders it inactive in the recombinant plasmid carrying it, which agrees with our observation that sialyltransferase activity cannot be detected in M982B cells. This frameshift is a deletion of a T residue, which is different from the G-tract frameshift mutations observed in the phase variable *lgt* genes from *N. meningitidis* and *N. gonorrhoeae* (8). This strain produces the same lacto-*N*-neotetraose as the L3 strains do but does not sialylate its LOS.[3] The acceptor specificity for the L3 enzyme with synthetic acceptors shows a strong preference for *N*-acetyllactosamine over lactose or galactose (Table II). Also the product of the reaction using enzyme from *N. meningitidis* and FCHASE-LacNAc acceptor was unequivocally determined by NMR to be FCHASE-α-2,3-sialyl-*N*-acetyllactosamine.

The expression level of the recombinant gene is being optimized, but it should be pointed out that the level of enzyme activity we have produced is approximately 50 units/liter of culture, based on assays with the FCHASE-LacNAc acceptor. Our expression levels are as high as those reported for mammalian sialyltransferases being overexpressed in insect cell cultures (18). We anticipate that optimization of expression of the *lst* gene in *E. coli* will yield substantially more enzyme activity than is produced with the mammalian gene constructs. This will be an important improvement for large scale chemienzymatic synthesis of α-2,3-sialylated oligosaccharides.

We have shown conclusively that we have cloned the α-2,3-sialyltransferase gene from the important mucosal pathogens *N. meningitidis* and *N. gonorrhoeae*. The availability of this gene will enable defined mutants to be constructed in both species in order to determine the role that LOS sialylation plays in the pathogenesis of these organisms, and the regulation of this important virulence factor can now be studied. The availability of large amounts of an α-2,3-sialyltransferase for enzymatic synthesis will help elucidate the role of this important modification of many oligosaccharide structures.

Acknowledgments—We thank D. W. Griffith for large scale production of *N. meningitidis* cells, Kirstin Tisdale for help in preparing samples for NMR, Jean-Robert Brisson for performing the NMR analysis, Joseph Michniewicz for the DNA sequencing, Doris Bilous for oligonucleotide synthesis, and C. Roger MacKenzie for critical reading of the manuscript.

REFERENCES

1. Harduin-Lepers, A., Recchi, M. A., and Delannoy, P. (1995) *Glycobiology* **5**, 741–758
2. Smith, H., Parsons, N. J., and Cole, J. A. (1995) *Microb. Pathog.* **19**, 365–377
3. Rest, R., and Mandrell, R. E. (1995) *Microb. Pathog.* **19**, 379–390

[3] H. J. Jennings, personal communication.

4. Aspinall, G. O., McDonald, A. G., Pang, H., Kurjanczyk L. A., and Penner, J. L. (1994) *Biochemistry* **33**, 241–249
5. Mandrell, R. E., Griffiss, J. M., Smith, H., and Cole, J. A. (1993) *Microb. Pathog.* **14**, 315–327
6. Mandrell, R. E., Smith, H., Jarvis, G. A., Griffiss, J. M., and Cole, J. A. (1993) *Microb. Pathog.* **14**, 307–313
7. Bramley, J., de Hormaeche, R. D., Constantinidou, C., Nassif, X., Parsons, N., Jones, P., Smith, H., and Cole, J. (1995) *Microb. Pathog.* **18**, 187–195
8. Jennings, M. P., Hood, D. W., Peak, I. R. A., Virji, M., and Moxon, E. R. (1995) *Mol. Microbiol.* **18**, 729–740
9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. MacKenzie, C. R., Sharma, V., Brummel, D., Bilous, D., Dubuc, G., Sadowska, J., Young, N. M., Bundle, D. R., and Narang, S. A. (1994) *Bio/Technology* **12**, 390–395
11. Tabor, S., and Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1074–1078
12. Wakarchuk, W. W., Martin, A., Jennings M. P., Moxon, E. R., and Richards, J. C. (1996) *J. Biol. Chem.*, **271**, 19166–19173
13. Pavliak, V., Brisson, J.-R., Michon, F., Uhrin, D., and Jennings, H. J. (1993) *J. Biol. Chem.* **268**, 14146–14152
14. Nakai, K., and Kanehisa, M. (1991) *Proteins Struct. Funct. Genet.* **11**, 95–110
15. Zhao, J. Y., Dovichi, N. J., Hindsgaul, O., Gosselin, S., and Palcic, M. M. (1994) *Glycobiology* **4**, 239–242
16. Ganguli, S., Zapata, G., Wallis, T., Reid, C., Boulnois, G., Vann, W. F., and Roberts, I. S. (1994) *J. Bacteriol.* **176**, 4583–4589
17. McLaughlin, R., Spinola, S. M., and Apicella, M. A. (1992) *J. Bacteriol.* **174**, 6455–6459
18. Williams, M. A., Kitagawa, H., Datta, A. K., Paulson, J. C., and Jamieson, J. C. (1995) *Glycoconjugate J.* **12**, 755–761